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Effects of Hyaluronic Acid on Mitochondrial Function and Mitochondria-driven Apoptosis following Oxidative Stress in Human Chondrocytes

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Hyaluronic acid is widely used in the treatment of osteoarthritis and exerts significant chondroprotective effects. The exact mechanisms of its chondroprotective action are not yet fully elucidated. Human articular chondrocytes actively produce reactive oxygen and nitrogen species capable of causing cellular dysfunction and death. A growing body of evidence indicates that mitochondrial dysfunction and mitochondrial DNA damage play a causal role in disorders linked to excessive generation of oxygen free radicals. We hypothesized that the chondroprotective effects of hyaluronic acid on oxidatively stressed chondrocytes are due to preservation of mitochondrial function and amelioration of mitochondria-driven apoptosis. When primary human chondrocyte cultures were exposed to reactive oxygen or nitrogen species generators, mitochondrial DNA damage along with mitochondrial dysfunction and mitochondria-driven apoptosis accumulated as a consequence. In addition, cytokine-treated primary human chondrocytes showed increased levels of mitochondrial DNA damage. Pretreatment of chondrocytes with hyaluronic acid caused a decrease of mitochondrial DNA damage, enhanced mitochondrial DNA repair capacity and cell viability, preservation of ATP levels, and amelioration of apoptosis. The results of these studies demonstrate that enhanced chondrocyte survival and improved mitochondrial function under conditions of oxidative injury are probably important therapeutic mechanisms for the actions of hyaluronic acid in osteoarthritis.

Intraarticular hyaluronan (HA)[2] therapy is used for the treatment of pain associated with osteoarthritis (OA) of the knee. As with all other available nonsurgical treatments for OA, HA is currently viewed as a treatment for only the symptoms of OA (1). However, the development of pharmacological treatments with the potential for structure-modifying activity in the treatment of OA, also called chondroprotective disease-modifying drugs for OA, has become a major focus in the field of OA research. Such compounds retard or stabilize the progression of established OA by altering the underlying pathological processes. There is a growing body of preclinical and clinical data, which suggests that intraarticular HA has disease-modifying activity, in addition to its proven efficacy and safety in treating the pain of OA patients. With the use of *in vitro* animal and human models, HA has been shown to exert a number of complex regulatory effects on the synovium, the articular cartilage, and the extracellular matrix of the knee joint (2). These effects include, but are not restricted to, influencing the synthesis of endogenous HA by synoviocytes (3), preventing the degradation of proteoglycan and collagen in the extracellular matrix (4), enhancing chondrocyte metabolism (5), inhibiting chondrodegeneration (6), preventing apoptotic death of chondrocytes (7), and inhibiting inflammatory responses that are associated with cartilage degradation (8).

It is well established that during the development of osteoarthritis, chondrodegenerative processes coexist with constant inflammatory/oxidative symptoms, and are both due to the destructive effects of reactive oxygen and nitrogen species (ROS and RNS), proinflammatory cytokines (*e.g.* interleukin-1β and tumor necrosis factor-α), and metalloproteinases, all substances that are produced in excess by the various joint cells in osteoarthritis (9, 10).

Oxidative stress, disrupted mitochondrial respiration, and mitochondrial damage have been found to promote aging, cell death, functional failure, and degeneration in a variety of tissues. Although oxygen diffuses into the articular cartilage and articular chondrocytes possess mitochondria and respire *in vitro*, a mitochondrially mediated pathogenesis for OA has not been studied extensively because articular cartilage chondrocytes must survive and maintain tissue integrity in an avascular and hypoxic environment, which requires adaptively increased anaerobic glycolysis to support ATP synthesis. However, mitochondrial oxidative phosphorylation may account for up to 25% of the ATP produced in cartilage (11). In addition, the direct, sublethal impairment of ATP production by chondrocyte mitochondria *in vitro* decreases matrix synthesis and increases matrix calcification (12, 13). Therefore, normal chondrocyte mitochondrial function is hypothesized to be essential for supporting ATP reserves in functionally stressed chondrocytes during the development of OA. Disruption of chondrocyte respiration by nitric oxide (NO), a mediator markedly up-regulated in OA cartilage, is centrally involved in functionally compromising chondrocytes (14). Furthermore, mitochondrial

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[2] The abbreviations used are: HA, hyaluronan; OA, osteoarthritis; XO, xanthine oxidase; ROS, reactive oxygen species; RNS, reactive nitrogen species; mtDNA, mitochondrial DNA; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; MOPS, 4-morpholinepropanesulfonic acid.

dysfunction is involved in NO-mediated apoptosis (15). In rat OA cartilage, as well as in human OA, mitochondria undergo ultrastructural changes that can be linked to different stages of cell death. Respiratory chain activity and mitochondrial membrane potential are significantly reduced in cultured human chondrocytes from patients with OA when compared with normal donors (16).

Each mitochondrion has its own genome. It is widely accepted that the mitochondrial genome is prone to oxidative damage, being 10–100-fold more sensitive than the nuclear DNA (17). Moreover, mutations and deletions in the mitochondrial genome have been linked to neurodegenerative disorders and other age-related diseases (18–20). Additionally, a growing body of evidence indicates that mtDNA damage could play a causal role in disorders linked to excessive generation of reactive oxygen species. Finally, there is evidence that suggests the involvement of mtDNA damage in the initiation of apoptosis (21–23). Based on these observations, the hypothesis tested in this study is that the chondroprotective action of hyaluronic acid on OA chondrocytes includes the prevention of mitochondrial dysfunction and mitochondria-driven apoptosis.

EXPERIMENTAL PROCEDURES

Cartilage Specimens and Chondrocyte Primary Cultures—For the current investigation, we used cartilage obtained from osteoarthritis patients after total knee replacement. Primary chondrocyte cultures from OA patients were generated by overnight digestion of minced cartilage samples with 5 mg/ml collagenase B (Roche Applied Science) in DMEM/F-12 supplemented with 10% FBS. Cells were plated on dishes in culture medium containing DMEM/F-12 with 50 ng/ml gentamycin and 10% FBS and used for experiments after reaching confluence (7–10 days). Primary chondrocyte cultures were never passaged in order to preserve chondrocyte phenotype. Furthermore, to ensure that collagenase did not cause a selective adverse affect in OA chondrocytes, we evaluated OA chondrocyte viability immediately after the overnight collagenase digestion using trypan blue dye exclusion and compared the results with the viability of normal chondrocytes obtained from normal donors following the same procedure. Viability following collagenase digestion was the same for both OA and normal specimens. Preliminary experiments showed that, following collagenase digestion, the viability of normal chondrocytes was $87 \pm 5\%$, whereas with OA, chondrocyte viability was $82 \pm 9\%$ ($n = 7$). Each separate experiment, including all of the necessary controls, was performed utilizing cultures produced from an individual cartilage specimen. Confluent cultures were routinely checked for the expression of collagen II and I by Western blot analysis with anti-collagen I and II antibodies (Gen Tech Inc.) to ensure that the chondrocytes studied had a normal phenotype. The ratio of collagen II/I for 36 analyzed samples was 396 ± 68.

Drug Preparation and Exposure—Primary chondrocyte cultures were exposed to different genotoxins for 30 min in DMEM/F-12 medium without FBS: 150, 300, and 600 μM of peroxynitrite (Cayman Chemicals); 10, 20, and 40 milliunits of XO plus 0.5 mM hypoxanthine (Sigma). Control cultures were exposed to DMEM/F-12 under the same condition. After 30 min, cells were lysed for dose-response experiments or rinsed and placed in normal culture medium to allow time for recovery and repair. Prior to treatment, some cells were incubated for 24 h with 500–730 kDa of sodium hyaluronate (Hyalgan) in concentrations ranging from 100 to 1000 μg/ml, with anti-CD44H antibody (BBA10; R&D Systems) or its matching epitope IgG 2A (R&D Systems) in a concentration of 5 μg/ml. For the combination of HA and antibody, cells were exposed for 6 h to antibody first and then to HA for 24 h prior to oxidative stress. The concentration of antibodies used was within the range used by other researchers for similar experiments (24, 25). Also, to mimic naturally occurring oxidative stress, primary human chondrocytes were preincubated with HA for 24 h and treated with 10 ng/ml of interleukin-1β or 50 ng/ml tumor necrosis factor-α (both from Roche Applied Science) for 48 h in DMEM/F-12 medium supplemented with 4% FBS. Following exposure or recovery, cells were collected and used for the evaluation of mtDNA repair and damage, ATP synthesis, and the induction of apoptosis evaluation or Western blot analysis.

Mitochondrial DNA and Repair Damage Assay—For DNA extraction, primary chondrocyte cultures were lysed in buffer containing 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 0.5% SDS, and 300 μg/ml proteinase K overnight. DNA was isolated by standard phenol/chloroform extraction and precipitated with cold ethanol. After overnight digestion with BamHI, each DNA sample was precisely quantified with a Hoefer DQ-300 fluorometer using Hoechst 33258 dye. Prior to loading on an alkaline agarose gel for Southern blot analysis, each sample containing 5 μg of total DNA was incubated with 0.1 N NaOH to reveal single strand breaks. After gel electrophoresis under alkaline conditions, DNA was transferred via vacuum transfer to a nylon membrane. Membranes were hybridized with a PCR-generated radioactive specific probe representing a part of the cytochrome *c* oxidase subunit III human mitochondrial gene. BamHI was selected because human mitochondrial DNA has a single restriction site for this enzyme, so that upon digestion it linearizes the mtDNA. Hybridization with the human mitochondrial gene-specific probe to cytochrome *c* oxidase subunit III recognizes a restriction band of 16,569 bp, corresponding to the whole mitochondrial genome. Autoradiograms were scanned for hybridization band intensity. DNA damage was evaluated as the amount of DNA breaks per 16.6-kb fragment. Break frequency was determined using the Poison expression ($s = -\ln P_0$, where s is the number of breaks per fragment, and P_0 is the fraction of fragments free of breaks). Subtracting the breaks at time t from the breaks present at 0 h and dividing by the 0 h breaks yielded the percentage of repair at time t. The resulting value then was converted to a percentage by multiplying by 100 (26).

Slot Blot Analysis—This assay was used to determine whether there were any differences in mtDNA content between different DNA samples. Different DNA samples obtained from normal donors and OA patients were digested with BamHI, precisely quantified, adjusted to the same concentration with H_2O, and treated with 0.3 M NaOH to denature the DNA. The fractions of 100, 50, and 20 ng were then blotted onto a nylon membrane (Zeta-probe GT from Bio-Rad) using a slot blot apparatus (Minifold II; Schleicher and Schuell), and mem-

branes were hybridized with an mtDNA-specific probe and washed according to the manufacturer's suggestions.

ATP Bioluminescence Assay—Nontreated and HA-pretreated human primary chondrocytes were exposed to different ROS/RNS generators (the same as for mtDNA damage studies) for 30 min and replaced with normal medium, and 3 h later, the ATP levels in cells were evaluated using an ATP bioluminescence assay kit (Roche Applied Science). This technique is well established and uses the ATP dependence of the light omitting luciferase-catalyzed oxidation of luciferin for the measurement of extremely low concentrations of ATP.

Nitric Oxide Release Assay—Total nitrite concentration in the tissue culture medium following cytokine treatment was measured in duplicate using a microplate assay employing the Griess reagent (0.5% sulfanylamide and 0.05% naphthalene diamine dihydrochloride in 2.5% orthophosphoric acid). One hundred microliters of this reagent were added to 100-μl aliquots of medium, and the optical densities were measured at 555 nm in a microplate reader after a 10-min incubation period at room temperature. Nitrite values were determined using sodium nitrite as the standard. Background nitrite values in the medium without cells were subtracted from values in the medium with cells.

Programmed Cell Death Evaluation—To evaluate if apoptosis is involved in chondrocyte death following ROS/RNS exposure, primary human chondrocyte cultures were exposed to 300 μM peroxynitrite or 20 milliunits of XO plus 0.5 mM hypoxanthine for 30 min. After treatment, the normal growth medium was replenished, and 24 h later, the appearance of apoptosis was evaluated by 4′,6-diamidino-2-phenylindole staining through the observation of condensed and fragmented nuclei. Ten fields containing ~100 cells were used to calculate the percentage of apoptotic cells.

Western Blot Analysis—To analyze changes in cell proteins, Western blot analysis was employed. For total cellular protein isolation, cells were lysed in cell lysing buffer (Cell Signaling Inc.) and processed according to the manufacturer's suggestions. These suspensions were briefly sonicated on ice and centrifuged once more at 5,000 × *g* to pellet any remaining debris, and the supernatant protein was used for Western blot assays. The protein concentration was determined using the Bio-Rad protein dye microassay according to the manufacturer's recommendation (Bio-Rad). For cytosolic protein isolation to perform the cytochrome *c* release assay, cells were harvested and treated with ice-cold digitonin (325 μM digitonin, 2.5 mM EDTA, 250 mM mannitol, and 17 mM MOPS, pH 7.4) for 80 s. The lysed cells were then added to mannitol-sucrose buffer to a final concentration of 1× (210 mM mannitol, 70 mM sucrose, 5 mM EDTA, pH 7.5). The suspension was then centrifuged for 10 min at 800 × *g* at 4 °C to pellet nuclei. The mitochondrial fraction was pelleted by centrifugation at 20,000 × *g* at 4 °C for 20 min. The resulting supernatant containing cytosolic proteins was centrifuged at 100,000 × *g* for 1 h at 4 °C to precipitate mitochondrial debris and membranes, concentrated using Amicon columns, and used for Western blot analysis.

Statistical Analysis—Statistical analyses were performed using either Student's *t* test or one- or two-way analysis of variance (GraphPad Prism) where appropriate. A difference of $p <$ 0.05 was considered significant. The Bonferroni *post hoc* test was used to determine the source of difference.

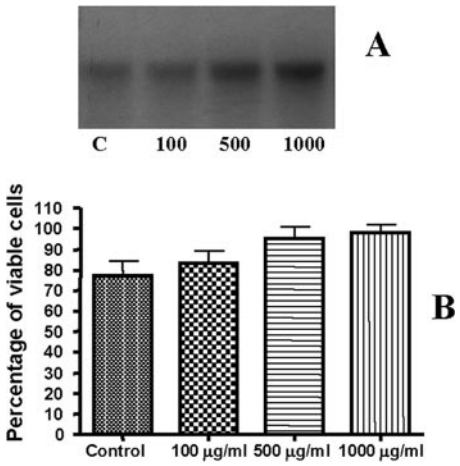

FIGURE 1. **HA protects OA chondrocytes from endogenous damage.** *A*, a representative autoradiogram from a Southern blot analysis of mtDNA from OA chondrocytes incubated for 24 h with 100, 500, and 1000 μg/ml Hyalgan. Human chondrocytes were lysed for DNA extraction following overnight digestion with collagenase B. The increased intensity of the hybridization bands indicates that endogenous mtDNA damage has been removed. *B* displays a comparison of the viability of OA chondrocytes with that of control following incubation with different HA doses for 24 h as assessed by trypan blue excision. The results were obtained from a minimum of eight independent experiments, and the values represent the mean percentage of viable cells ± S.E. Each independent experiment was performed utilizing cultures obtained from an individual cartilage specimen.

RESULTS

To ensure that HA itself did not cause mtDNA damage to chondrocytes, initial dose-response experiments were performed. Confluent primary cultures of chondrocytes were incubated for 24 h with increasing doses of HA (Hyalgan) ranging from 100 to 1000 μg/ml. Following this time, cells were lysed, and total DNA was isolated and subjected to quantitative Southern blot analysis. Interestingly, we observed increased hybridization band intensity in DNA samples from cells pretreated with HA, suggesting that hyaluronic acid protects OA chondrocytes from endogenous mtDNA damage that occurs normally in the mitochondria from OA chondrocytes (Fig. 1*A*). To ensure that differences between hybridization band intensity were due to increased mtDNA integrity and not mtDNA copy number, slot blot analysis of all of the samples was performed. This analysis revealed that there was no difference in the amount of mtDNA between DNA samples from control cells and cells treated with increased HA doses (data not shown). Also, increased cell viability following incubation with HA was observed (Fig. 1*B*). An HA concentration of 500 μg/ml was selected for subsequent experiments, since optimal protective effects were observed at this concentration.

Our main goal was to evaluate the protective effects of HA on chondrocytes following experimental stress induced by ROS or RNS, which simulates the increased NO and RNS production during OA progression. To study this, primary human chondrocyte cultures obtained from OA patients were subjected to oxidative stress induced by 150, 300, and 600 μM of peroxynitrite (RNS), or 10, 20, and 40 milliunits of xanthine oxidase with 0.5 mM hypoxanthine (ROS). Prior to genotoxin exposure,

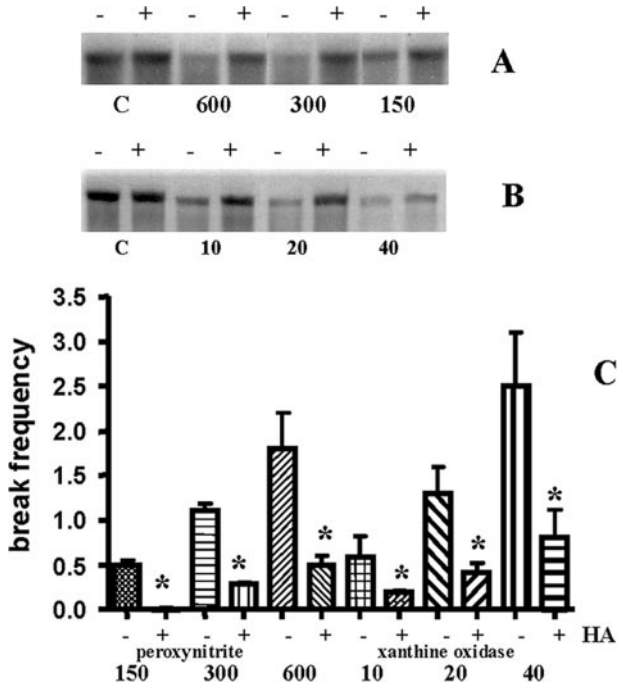

FIGURE 2. **HA protects mitochondrial DNA from OA chondrocytes from oxidative stress caused by exposure to ROS or RNS.** Chondrocytes were exposed to increasing concentrations of 150, 300, and 600 μM peroxynitrite or 10, 20, and 40 milliunits/ml xanthine oxidase with 0.5 mM of hypoxanthine for 30 min. Some cells were preincubated with 500 μg/ml of HA for 24 h prior to treatment. Cells were lysed, and DNA was isolated and subjected to Southern blot analysis. *A*, a representative Southern blot analysis of the effect of HA on peroxynitrite-induced mtDNA damage in chondrocytes; *B*, the effect of HA on xanthine oxidase-induced mtDNA damage, *C*, quantitation of mtDNA damage. *Plus* and *minus symbols* indicate the presence or absence of HA. An increase in the break frequency measured by analysis of Southern blots indicates that more mtDNA damage has accumulated. The results were obtained from a minimum of six independent experiments, and the values represent the mean break frequency ± S.E. *, a significant difference ($p < 0.05$) in damage in mtDNA in chondrocytes preincubated with HA as compared with untreated controls. Each independent experiment was performed utilizing cultures obtained from an individual cartilage specimen.

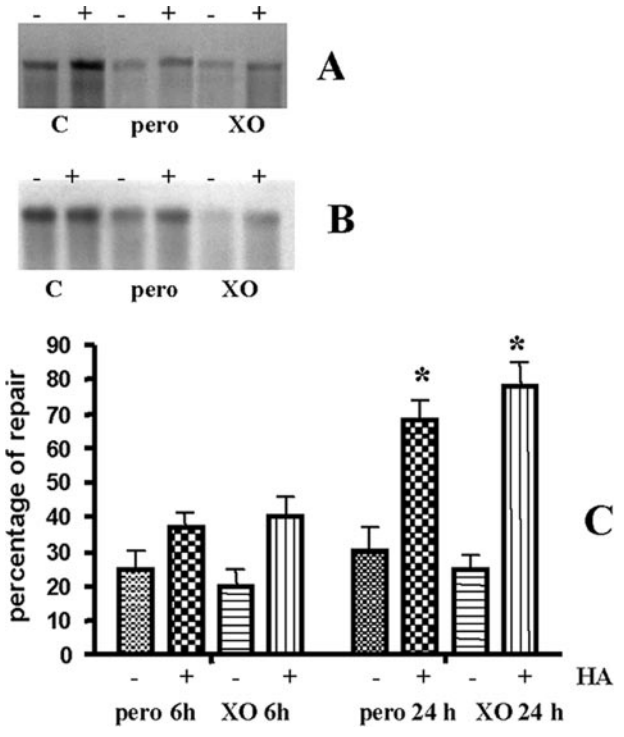

FIGURE 3. **HA enhances mtDNA repair in OA chondrocytes.** HA-preincubated and untreated primary human chondrocytes from OA patients were exposed to 300 μM peroxynitrite or 20 milliunits of xanthine oxidase, 0.5 mM hypoxanthine for 30 min and allowed to repair for 6 and 24 h. HA-preincubated cells received the same amount of HA in the recovery medium. Cells were lysed, and DNA was isolated and subjected to Southern blot analysis of mtDNA repair following 6 h of drug exposure. *B*, mtDNA repair following 24 h of drug exposure. *C*, a calculation of the percentage of repair measured by the change in break frequency determined by Southern blot analysis. *Plus* and *minus symbols* indicate the presence or absence of HA. The results were obtained from a minimum of seven independent experiments, and the values displayed represent the mean break frequency ± S.E. *, a significant difference ($p < 0.05$) between HA-treated and nontreated chondrocytes. Each independent experiment was performed utilizing cultures obtained from an individual cartilage specimen.

some cells were pretreated with hyaluronic acid (Hyalgan) at a concentration of 500 μg/ml for 24 h. Cells were treated with peroxynitrite or xanthine oxidase for 30 min and used immediately for dose-response experiments or replenished with normal growth medium and allowed to recover and repair for 24 h. HA-pretreated cultures were maintained in medium that also contained HA. The results revealed that HA protected chondrocyte mitochondrial DNA from damage induced by reactive oxygen and nitrogen species (Fig. 2). Similar results were obtained when the effects of hyaluronic acid on mtDNA repair capacity were studied. Concentrations of 300 μM peroxynitrite and 20 milliunits of XO were selected for repair experiments because these concentrations caused sufficient mtDNA damage for study, and also more than 50% of the cells still remained viable. Human chondrocytes, which were pretreated with hyaluronic acid for 24 h and had hyaluronic acid in the repair medium, had a significant improvement in the removal of DNA lesions after oxidative stress was introduced (Fig. 3). The enhancement of mtDNA repair directly correlated with increased chondrocyte viability. Pretreatment with HA and the addition of HA to the culture medium of HA-pretreated cultures during recovery from oxidative stress significantly improved viability of OA chondrocytes (Fig. 4).

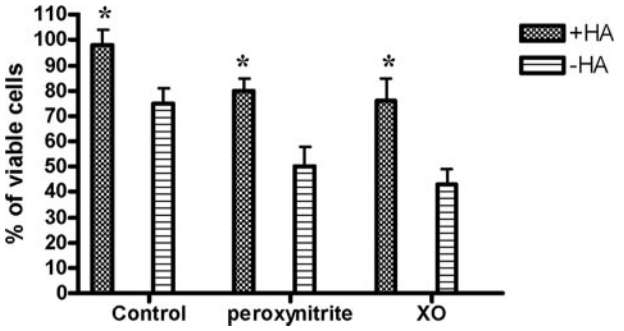

FIGURE 4. **HA enhances chondrocyte viability following exposure to ROS or RNS.** HA-pretreated and untreated confluent cell cultures were exposed to 20 milliunits/ml xanthine oxidase, 0.5 mM hypoxanthine or 300 μM peroxynitrite for 30 min and allowed to recover for 24 h. HA-preincubated cells received the same amount of HA in recovery medium. After 24 h, cells were trypsinized away from the culture vessel, incubated with trypan blue, and viewed by light microscopy to calculate the number of viable and dead cells. The results were obtained from a minimum of six independent experiments, and the values represent the mean percentage of viable cells ± S.E. *, a significant difference ($p < 0.05$) in HA-pretreated OA chondrocytes as compared with those that did not receive HA. Each independent experiment was performed utilizing cultures obtained from an individual cartilage specimen.

One of the most important mitochondrial functions is the production of energy by the generation of ATP. For the current studies, we investigated whether hyaluronic acid had any effect

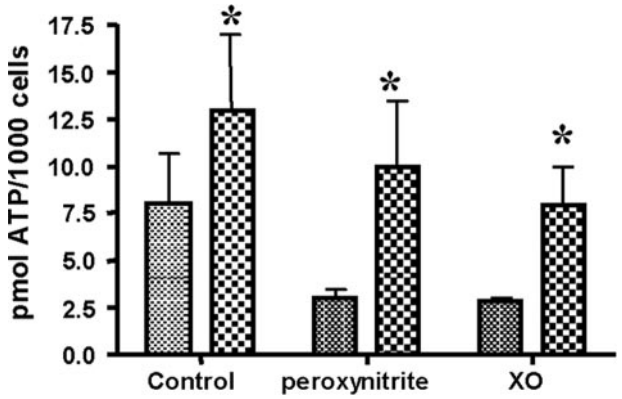

FIGURE 5. **HA preserves ATP levels in primary chondrocytes from OA patients following exposure to ROS/RNS generators.** HA-pretreated and nontreated confluent cell cultures were exposed to 20 milliunits/ml xanthine oxidase plus 0.5 mM hypoxanthine or 300 μM peroxynitrite for 30 min and incubated for an additional 3 h in normal culture medium. Following that time, cells were lysed and subjected to an ATP bioluminescence assay. Values represent the mean ± S.E. from six independent experiments. *, significant difference ($p < 0.05$) from control. Each independent experiment was performed utilizing cultures obtained from an individual cartilage specimen.

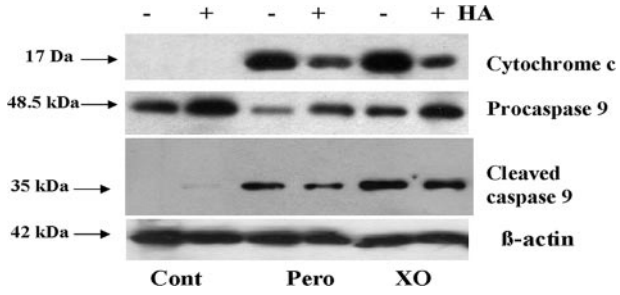

FIGURE 6. **HA protects OA chondrocytes from mitochondria-driven apoptosis.** HA-pretreated and nontreated confluent cell cultures were exposed to 20 milliunits/ml xanthine oxidase plus 0.5 mM hypoxanthine or 300 μM peroxynitrite for 30 min and then incubated for an additional 24 h in normal culture medium. HA-preincubated cells had the same amount of HA in culture medium. Western blot analysis was performed on total cytosolic protein fractions using antibodies against cytochrome *c* and procaspase or cleaved caspase 9. Antibody against β-actin was used to ensure equal loading of samples.

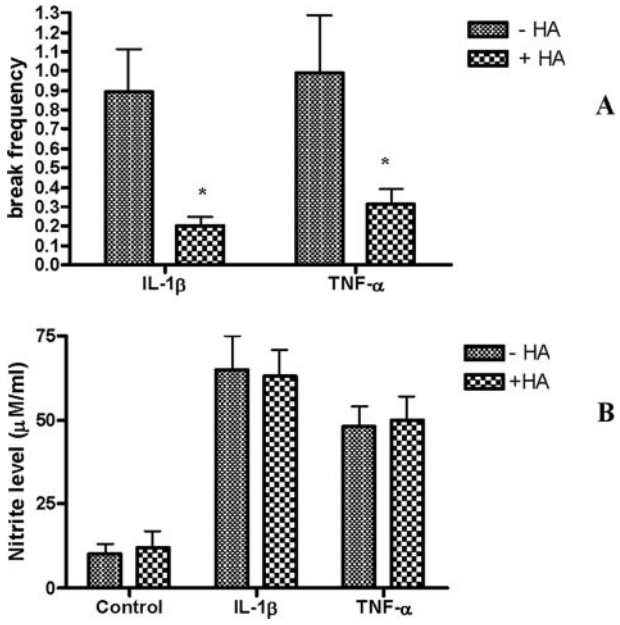

FIGURE 7. **HA protects chondrocytes from cytokine-induced mtDNA damage.** OA chondrocytes were incubated for 48 h with 10 ng/ml IL-1β or 50 ng/ml tumor necrosis factor-α alone or in combination with 500 μg/ml HA in DMEM/F-12 medium supplemented with 4% FBS. *A* represents the accumulation of mtDNA damage determined by the increase in break frequency as measured by Southern blot analysis. *B*, evaluation of nitrite levels following cytokine treatment or combination of cytokines and HA. The results were obtained from a minimum of six independent experiments, and the values represent the mean break frequency ± S.E. *, a significant difference ($p < 0.05$) between HA-treated and nontreated chondrocytes. Each independent experiment was performed utilizing cultures obtained from an individual cartilage specimen.

on ATP levels following oxidative stress. Confluent primary cultures of chondrocytes were treated with peroxynitrite or xanthine oxidase as described above, and 3 h later, ATP levels were analyzed using an ATP bioluminescence assay. Hyaluronic acid had a protective effect on ATP levels, and this may be one of the predominant mechanisms of its chondroprotective effects (Fig. 5).

Previous concepts of OA pathogenesis have focused on the role of chondrocytes in the degradation of the extracellular matrix. More recent findings suggest that chondrocyte death and survival are closely linked to cartilage matrix integrity. Chondrocyte apoptosis has been described in both human and experimentally induced OA, with nitric oxide considered as the primary inducer of this process. To evaluate whether ROS/RNS exposure leads to apoptosis and if HA has any effect on programmed cell death, primary cultures of human chondrocytes were exposed to peroxynitrite and XO with hypoxanthine in the same conditions as were described in the preceding studies. After treatment, the normal growth medium was replenished, and 24 h later, the appearance of apoptosis was evaluated using 4′,6-diamidino-2-phenylindole staining to identify condensed and fragmented nuclei. Both peroxynitrite exposure and XO/hypoxantine treatment caused apoptosis to appear, and hyaluronic acid had a protective effect against this apoptotic cell death. Following peroxynitrite treatment, 28 ± 5% of cells were found to be apoptotic, whereas HA reduced this number to 7 ± 2% of cells. After exposure of chondrocytes to XO, 35 ± 7% of cells became apoptotic, whereas the addition of HA lowered the proportion of apoptotic cells to 9 ± 3%. In control cells, 7 ± 1% of chondrocytes were found to be undergoing apoptosis, and after exposure to HA, this percentage dropped to 4 ± 1%. To evaluate the mitochondrial involvement in the initiation of apoptosis, cytochrome *c* release from mitochondria into the cytosol along with caspase 9 activation were studied. The cytochrome *c* release assay showed that apoptosis in chondrocytes following ROS/RNS exposure involves mitochondrial dysfunction (see Fig. 7). The caspase 9 cleavage assay revealed that there was a significant decrease in procaspase 9 levels and a concomitant increase in the large 35-kDa cleaved caspase 9 fragment following exposure to peroxynitrite and xanthine oxidase, and this effect was reversed by the addition of HA (Fig. 6).

The up-regulation of proinflammatory cytokine expression is considered to be the main cause of RNS/ROS production during the development of joint disease. Therefore, mtDNA integrity and the effects of HA were examined by Southern blot analysis following exposure of human chondrocytes to cytokines (Fig. 7*A*). The results revealed that mtDNA was damaged by both interleukin-1β and tumor necrosis factor-α, and HA

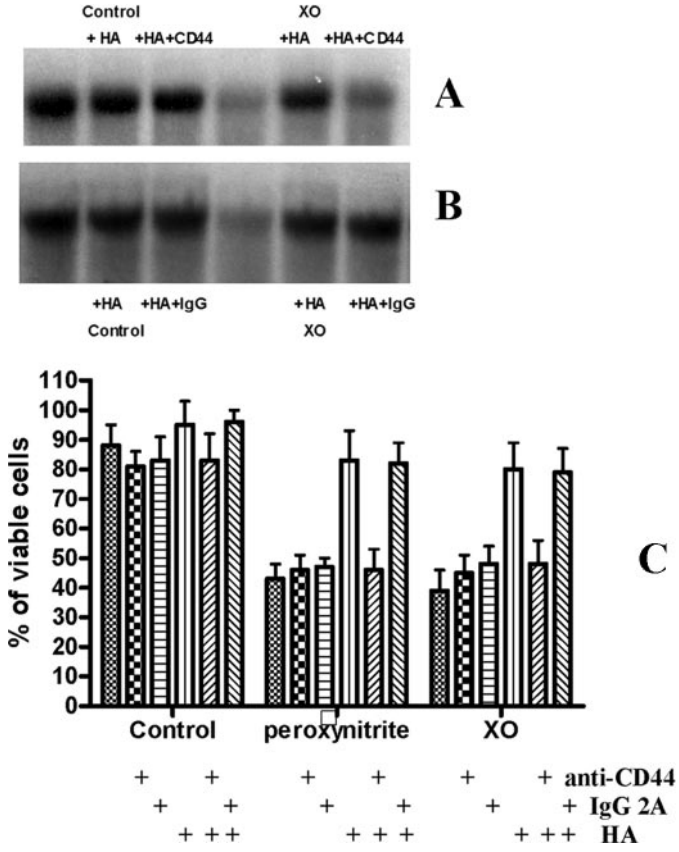

FIGURE 8. **Anti-CD 44H antibody abolishes the protective effects of HA on OA chondrocyte mtDNA integrity and viability.** Chondrocytes were exposed to 300 μM peroxynitrite or 20 milliunits/ml xanthine oxidase with 0.5 mM hypoxanthine for 30 min. Some cells were preincubated at first alone or with 5 μg/ml anti-CD44H antibody or IgG 2A matching epitope antibody for 6 h following incubation with 500 μg/ml HA for 24 h prior to treatment. Cells were lysed, and DNA was isolated and subjected to Southern blot analysis. As can be seen in *A*, anti-CD44 abolished the protective effect of HA on mtDNA damage induced by XO/hypoxanthine. *B* shows that there was no effect of IgG 2A antibody on protective properties of HA on primary chondrocytes. *C* displays the viability of chondrocytes pretreated with HA, anti-CD44H, IgG 2A, HA plus anti-CD44H, and HA plus IgG 2A following induction of oxidative stress. The results were obtained from a minimum of six independent experiments, and the values represent the mean percentage of viable cells ± S.E.

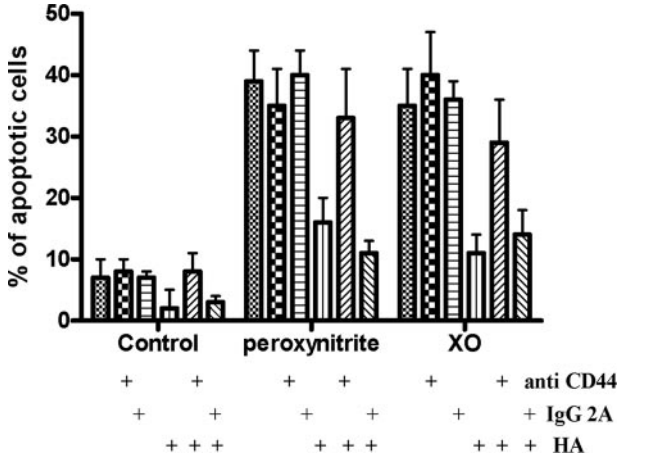

FIGURE 9. **The effect of HA and anti-CD44 on the accumulation of apoptotic chondrocytes following exposure to ROS or RNS.** Chondrocytes were exposed to 300 μM peroxynitrite or 20 milliunits/ml xanthine oxidase with 0.5 mM hypoxanthine for 30 min and then cultured for an additional 24 h. Some cells were preincubated at first alone or with 5 μg/ml anti-CD44H antibody or IgG 2A matching epitope antibody for 6 h following incubation with 500 μg/ml HA for 24 h prior to treatment. HA-preincubated cells had the same amount of HA in culture medium. Cells were washed three times with PBS and fixed with 4% paraformaldehyde. The fixed cells were washed again with PBS and stained with 1 μg/ml 4′,6-diamidino-2-phenylindole. Stained cells were examined by fluorescent microscopy to identify apoptotic cells. To evaluate the percentage of apoptotic cells, 6–7 fields from each experimental condition were viewed in a blinded fashion until a total of 500 cells were observed. The results were obtained from a minimum of six independent experiments, and the values represent the mean percentage of apoptotic cells ± S.E. Each independent experiment was performed within cultures obtained from an individual cartilage specimen.

was able to ameliorate this damage. To investigate whether HA could prevent mtDNA damage during cytokine exposure by inhibiting NO production, nitrite levels were evaluated using the Griess reaction. The results showed that HA had no significant effect on nitrite levels (Fig. 7*B*).

To evaluate whether the protective effects of HA were mediated through HA-CD44 interactions, chondrocytes were preincubated alone or with anti-CD44H or its matching epitope IgG 2A antibody for 6 h following 24 h of HA incubation and then subjected to oxidative stress. Following ROS/RNS exposure and recovery, cells were evaluated for mtDNA damage, viability, and apoptosis. As can be seen in Figs. 8 and 9, anti-CD44H antibody binding to HA abolished its protective effects against mtDNA damage, loss of viability, and induction of apoptosis. IgG 2A antibody did not modify any of the protective effects of HA on human chondrocytes.

DISCUSSION

The principle finding of this investigation is that an essential mechanism for the chondroprotective actions of hyaluronic acid on human chondrocytes is the preservation of mitochondrial function. Incubation of primary human chondrocytes obtained from OA patients with HA leads to increased mtDNA integrity, improved ATP levels, and increased cell viability when cells were cultured under normal conditions. Similar results were observed when an oxidative insult was induced by either peroxynitrite or xanthine oxidase/hypoxanthine. HA ameliorated the negative effects of reactive oxygen and nitrogen species on mtDNA integrity, mtDNA repair, ATP production, and cell viability. HA also had an effect on apoptosis induced following oxidative stress. HA treatment decreased the number of apoptotic cells and the release of cytochrome *c* from chondrocyte mitochondria and blocked the cleavage of procaspase 9. Also, the protective effects of HA were observed against the mtDNA damage, which accumulates following proinflammatory cytokine exposure. Anti-CD 44 antibody at saturating concentrations abolished these protective effects of HA.

Hyaluronic acid, one of the chief components of the extracellular matrix, is a glycosaminoglycan that has a variety of effects on cells, including the suppression of cartilage matrix degradation and increasing cell proliferation. In arthritic disease, the concentration and molecular weight of hyaluronan decreases, compromising the viscoelastic properties of joint fluid. Therefore, it is essential to approach this orthopedic disease with treatments that not only protect the cartilage against degenerative damage by stimulating its intrinsic repair capacity but also neutralize the inflammatory/destructive potential of the mediators involved in the oxidative-inflammatory process. In the present study, we show that hyaluronic acid has the capacity to protect mitochondria and its genome from the dam-

aging effects of oxidative stress and preserve one of the most essential mitochondrial functions, energy production. To the best of our knowledge, this is the first report that demonstrates the mitoprotective and genoprotective effects of HA. Additionally, the present study revealed that one of the protective functions of HA is the enhancement of cell survival. HA not only increased the viability of normal chondrocytes but also enhanced the survival of cells damaged by ROS/RNS. These data are in agreement with evidence provided by other authors about the effects of HA on chondrocyte viability (27, 28).

Despite the confusion that still exists with respect to the physiological role of HA and its receptors and the influence of molecular weight on its pharmacological activity, it is clear that HA, after binding to the cell surface, triggers a cascade of cellular events, such as growth, differentiation, and migration. HA also appears to have an important role in scavenging oxygen radicals (29). Additionally, most of the endogenous effects of HA appear to be mediated through CD 44 receptor binding (30, 31). There is a large body of evidence that indicates that the CD44 receptor may play a critical role in the normal function and survival of many cell types. CD44 can promote resistance to apoptosis in colonic epithelium via a mitochondria controlled pathway (32) and block stress-induced, p53-dependent cytostatic and apoptotic signals in untransformed cells (33). Moreover, it critically supports tumor growth and metastasis in experimental models of solid cancers (34). Additionally, it has been shown that expression of CD44 in some cell types, such as stem cells, may provide the means to internalize HA by endocytosis, and one of the functions of internalized HA may be protection of DNA from oxidants (35). The mechanism for the protective effect of HA may involve either entrapment of iron ions, thereby inhibiting the Fenton reaction that produces secondary oxidative species, or directly scavenging of primary and secondary ROS as an antioxidant. However, information is sparse concerning how exactly CD44 may affect mitochondrial function other than by interacting with the mitochondrial pathway for apoptosis. In relationship to cartilage biology, it has been shown that CD44 plays an important role in both the normal and abnormal function of cartilage through its adhesion to HA, which induces a variety of stimulatory signals that regulate chondrocyte proliferation as well as matrix synthesis in the cartilage microenvironment (36).

We believe that our findings are unique in that they show that CD44-HA interactions affect mitochondria in chondrocytes and contribute to clarifying the role of HA and CD44 in chondrocyte function and survival. Under conditions of oxidative stress or any other stress, cells respond by employing multiple protective mechanisms. Mitochondria are very sensitive to stress and can be easily damaged as a consequence. We recently showed that mtDNA integrity and repair are affected during the development of OA (37). We do not know at this point whether the effects of HA that we observed are due to the direct presence of HA in mitochondria. Additional investigation will be required to clarify this point. Because CD44-HA interactions were found to be important for the effects of HA on mitochondria, it is possible that the protective effects of HA are indirect and due to regulatory changes in chondrocyte function, such as global stress responses. In the present study, we obtained mixed results in relationship to the direct antioxidant scavenging activity of HA against ROS/RNS and its regulatory effects mediated by CD 44 binding. HA protected mtDNA against the initial damage introduced by free radicals, which was a direct antioxidant response. At the same time, anti-CD 44 antibody ameliorated the protective effects of HA on cell viability and apoptosis. These findings support both the complexity and multiplicity of HA actions on cellular function. Clearly, more studies are warranted to completely understand the mechanisms by which HA protects chondrocytes and to maximally exploit its therapeutic potential in OA. In conclusion, our results demonstrate that enhanced chondrocyte survival and improved mitochondrial function under conditions of oxidative injury are probably important therapeutic mechanisms for the actions of HA in osteoarthritis.

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